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VITAL STAINING OF HUMAN BLOOD WITH SPECIAL REFERENCE TO THE SEPARATION OF THE MONOCYTES*

BY

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The possible differing behavior of blood cells to vital stains has not yet been adequately explored. Yet if one will search carefully the literature of vital staining it will be seen that Certes, Mitrophanow, Teichmann, Galeotti, Ehrlich and Muller, Arnold, and Plato, to name no others among the earlier observers, subjected the leucocytes to some scrutiny in experiments with vital dyes. Furthermore, during the last ten years two or three significant attempts have been made to investigate blood cells by the supra-vital application of dyes. I refer especially to the papers of Rosin and Bibergeil, Hammar, Cesaris-Demel, and Antonio Ferrata. This work has made surprisingly little impression on hematology. It is lacking chiefly, perhaps, in not being essentially systematic. Hitherto no attempt has been made to show the characteristic behavior of living blood cells toward dyes representative of the different dye groups, nor has there been sufficient study of the differential behavior of the various types of blood cells toward any one dye substance. We are not without justification, however, in hoping that such studies will throw light on the physiology of the leucocytes and above all on interrelationships between the various white blood cells. These studies, begun in the Anatomical Laboratory of the University of California in 1917 by M. C. Silverberg and A. C. Silbermann, were carried further in the next succeeding years by Robert T. Trotter. At the suggestion of Professor Evans and with his aid, the present writer has within the last two years attempted to make a comprehensive study of the behavior of living human blood

* This paper and the following one are preliminary accounts of work accomplished during the last two years and described in full in theses formally filed with the Dean of the Graduate School of the University of California on September 6, 1921. The complete illustrated publications will appear elsewhere.

cells when subjected in thin films to the action of representative organic dyestuffs. The paper is one of a series on the reaction of the tissues under normal and pathological conditions studied by means of vital stains. About one hundred and fifty dyes of known chemical constitution were compared in respect to their action on living leucocytes. The dyes were representative of all of the dye families.

The method employed was one which has been ascribed to Rosin and Bibergeil (1902–04), but in its use Pappenheim disputes the priority.[1]

The end of a slide which has been dipped in an alcoholic solution of the dye is drawn across the warm surface of another slide. This leaves a thin film of solid dye on the second slide, a film consisting of very minute particles which will redissolve rapidly. A small drop of blood is then placed on a cover slip, and the cover slip allowed to come in contact with the slide. If the glassware is scrupulously clean and possesses an even surface, the blood will spread evenly and quickly to the margin, or better, almost to the margin of the cover slip. The cover slip is then quickly rimmed with paraffin or lanolin. The whole procedure must be carried through in a few seconds after obtaining the fresh drop of freely flowing blood. For making the dye "films," solutions of varying concentration should be employed. A 1 per cent alcoholic solution of the dye was the concentration systematically tried first. Then, judging by the results obtained, lower and lower concentrations were employed. At times even a .001 per cent solution gave results. Such beautiful preparations supply one with a film consisting of a single layer of living blood cells spread out in the most advantageous position for study. With experience injury to the cells from handling is minimal, and in totally unstained preparations or in those made with very weak solutions of many dyes, living healthy cells may be observed pushing out pseudopodia or moving in amoeboid fashion for several hours. Illumination through Gage's daylight glass and apochromatic oil lenses were employed.

Dyes having the greatest differences in chemical and physical properties may enter and may be stored by the living blood cells. As regards chemical make-up of the dyes, the following table will give the number of representatives of the various dye groups which were employed, and their positive or negative behavior as supra-vital stains.

[1] For further discussion of the priority in the use of this method see the discussion by Hammar (1912) and by Pappenheim (1907).

Dye group	Positive*	Negative*
Nitroso	....	1
Nitro	1	....
Stilbene	2	1
Pyrazolen	....	1
Monazo	....	6
Disazo	3	4
Triphenylmethane	2	17
Xanthones	6	7
Acridines	1	1
Oxazine	13	8
Thiazine	5	5
Azine—		
(*b*) Eurhodine	1	....
(*c*) Aposafranine	....	2
(*d*) Benzosafranine	19	5
2. Naphtosafranine	1	2
(*e*) Induline	1	2
Anthraquinone	2	10
Indigo	2	1
Inorganic	2	....
Totals	81	73

* "Positive" means that the dye was observed in the cytoplasm of cells either diffusely, in the specific granules, in mitochondria or in special storage granules, before the nucleus stained. "Negative" means either that the dye was not seen to penetrate the cell at all, or that if it did appear in the cell, death of the cell was coincident with the entrance of the dye.

The supra-vital dyes may stain structures which existed in the cell at the time of treatment with the dye and which are therefore comparable to structures stained in fixed preparations. One may therefore obtain satisfactory vital stains of the specific granules of the polymorphonuclear leucocytes. Supra-vital methods may indeed produce the most striking stains of the specific granules. I may enumerate here Victoriablau B, Victoriablau 4R, Neumethyleneblau N, Neumethyleneblau GG, Neuechtblau F, Nile Blue R, Nile Blue A, Nile Blue B extra, Nile Blue BB, Methylene Blue Med., Capri Blue GON, Brilliant Cresyl Blue, Thionin Blue GO, Toluidin Blue, Anthrachinongrun GXNO, Brilliantrhodulin Red B, Indazine M.

Very few instances, however, were found of dyestuffs which stained these structures alone, and in those instances the dyes employed could not be called good vital stains. The above mentioned dyes, besides staining specific granules, may stain beautifully the "segregation" granules, to be mentioned later.

As Laguesse, Michaelis, Bensley, and Cowdry have shown, mitochondria may be electively stained by vital dyes. The best example of such a dye is Janus Green B, to which it is now possible to add Amethyst Violet, Iris Violet, Janus Grey 2B, Janus Dark Blue R, Diazine Black, Janus Black II, Naphtindon, Echtneutralviolett.

The mitochondrial content of the mononuclear cells is higher than that of the polymorphonuclear cells. Among the polymorphonuclear cells the mitochondria are more numerous and easier to demonstrate in the neutrophils than in the eosinophils. The basophils are too infrequent in human blood to be placed readily in such a series. From work on the rabbit, however, where basophils are abundant, the mitochondrial content of the basophils has been shown to be very low. In the mononuclear cells the number of mitochondria present seems to be a function of the amount (size) of the cytoplasm, being invariably numerous in the transitionals and always more numerous in the larger than in the small lymphocytes.

The rich mitochondrial content both of large lymphocytes and of monocytes makes it impossible by this means to discriminate between the lymphocytes and the other important group of mononuclear cells to which the name monocyte has now been given (Naegeli). Fortunately, in the case of other vitally stained granules—the segregation granules—such a distinction between lymphocytes and monocytes is now possible, as will be described later.

The lymphocytes are particularly well adapted for the observation of mitochondria in fresh blood preparations, not only because they are numerous in these cells but because of the almost complete absence of other granules. Only an occasional refractive vacuole and two or three "segregation granules" are present.

Cowdry (1914, 1916) has spoken of the specificity of the staining of mitochondrial substance to the safranine chemical nucleus and also of the importance of the substituted groups. In this study the safranine derivatives were found to include all the best mitochondrial stains. However, dyes representative of at least three Schultz groups were found to contain mitochondrial stains. It is therefore evident that the safranine nucleus can not be regarded as a specific requirement for mitochondrial stains. The importance which has been placed on the ethyl group in mitochondrial stains has also probably been overestimated. This seems to be true even in the azine group (safranine nucleus) where the ethyl group has been considered by Cowdry (1916) to be the factor which allowed Janus Green B to stain mitochondria while Janus Green G, of the same constitution except in this substitution of the two ethyl groups of Janus Green B by methyl groups, was not a mitochondrial stain. Naphtindon (Safranine-Beta Naphthol) is an example of a mitochondrial stain which contains neither ethyl nor methyl groups. In other dye classes containing mitochondrial stains, some of the dyes contain only methyl groups (two, four,

or six) substituted for the hydrogen of the amine groups, others contain only ethyl groups, and still others contain both groups. It would therefore seem that these groups are not specific for the mitochondrial reaction.

Many dyes are attracted to and concentrated in a special set of granules and vacuoles in certain cells, structures to which Evans and Scott have given the term "segregation apparatus," and which are largely, although not exclusively, preformed structures. These structures are present in blood cells. They may be brilliantly displayed by vital stains. The vacuoles often have the power to increase in size as the amount of the dye stored in them increases. This reaction on the part of the living cell may be regarded as a special contrivance to isolate from the protoplasm and hence render harmless either excretory products or foreign materials forced on the cell. The reaction is given best perhaps by Neutral Red, Nile Blue Sulfate, and Brilliant Cresyl Blue, but over a score of other dyes were found to behave typically in this way.

Ferrata, Rosin and Bibergeil, Dubreuil, Cesaris-Demel, and Hammar have especially studied the granules which may be produced in blood cells by the application of this group of dyes. In order to justify a term like "segregation apparatus," it is necessary to separate these granules from the specific granules and from the mitochondria; this distinction can be made. Both the size, shape, and number of the granules are good criteria for the differentiation of the segregation apparatus from mitochondria. With dyes like Janus Green B and Neutral Red the granular, rod, and filamentous forms of the mitochondria are especially contrasted with the globules of the segregation apparatus because of the peculiar tendency of the latter structures to increase rapidly in size. Yet it would not be justifiable to assume that all globular structures are segregation bodies and all filamentous forms mitochondria. The best mitochondrial stains, including Janus Green B, eventually distort the mitochondrial morphology, and they may do this very rapidly if high concentrations are employed. In the neutrophils, eosinophiles, and monocytes the difference in the number of granules in the segregation apparatus and the number in the mitochondrial apparatus is not a conspicuous feature. It is in the lymphocytes that there is a marked difference in the number of the two sets of granules. Double staining, for example, with Neutral Red and Janus Green well illustrates this difference. In the case of the lymphocytes, the contrast between the few red granules of

the segregation apparatus (from one to three or four) and the numerous mitochondria (from ten to twenty or more) is always demonstrable.

The low content of lymphocytes in segregation granules and the exceptionally high content of monocytes enables us to separate these cells. The classification of the mononuclear cells of the blood has been a source of dispute among hematologists for years. Agreement was easily reached on the lymphatic origin of the small cells. It was not so simple a matter, however, to decide on the origin and relationships of the larger mononuclear cells. The early classifications of the mononuclear cells were based on morphology and fixed staining reactions and led to widely different ideas of the relationships of these cells. Ehrlich separated them into lymphocytes, large mononuclears, and transitionals. The latter two groups represented, according to Ehrlich, different stages of conversion from lymphocytic to the polymorphonuclear cells. Weidenreich saw among the mononuclears only one type of cell, the lymphocyte. The introduction of biological criteria has not lessened the complexity of the subject. Naegeli (1919), on the basis of morphological staining and biological reactions, divides the mononuclears into two classes, the lymphocytes and the monocytes (including the large mononuclears and transitionals of Ehrlich). Monocytes are by him supposed to constitute an independent group of cells of myeloid origin. His morphological criteria for recognizing these cells consist in their possession of a characteristic, abundant, fine, dustlike granulation stained by Azur and the fact that these cells give a positive oxydase response. Biological affiliations are shown by cases where stimulation of myeloid cells also leads to stimulation of the monocytes, and cases where inhibition of these cells also inhibits the monocytes, while stimulation of lymphatic tissue does not influence the monocyte count. McJunkin (1919) has also applied morphological, staining, and biological methods and draws widely different conclusions; but although McJunkin, with Mallory and others, classifies these questionable large mononuclears of the normal blood as derivatives of endothelium and uses the term "endothelial leucocytes" to designate them, sufficient evidence to justify this term has not been produced. In some cases he used a modified oxydase method for recognizing the cells.[2] McJunkin's biological test consists in determining

[2] The application of the oxydase as a method for separating these cells in some mammals has proved exceedingly disappointing. Equally capable hematologists report a negative as well as a positive response on the part of the transitionals or monocyte cell.

phagocytic function under a definite set of conditions. He finds one set of conditions under which the vascular endothelium and these large mononuclear cells of the blood are the only cells which phagocytize carbon, following intravenous carbon injections, and he deduces that the transitions are derivatives of this endothelium. Furthermore, while there could be no doubt that the transitional leucocytes are very much more active phagocytes than are the lymphocytes, and in these respects are like the specific endothelia, it is unlikely that one can produce conditions which will force every transitional to exhibit this activity. At any rate, in these particular experiments McJunkin used no other criteria save carbon injection to recognize the transitional cells, and admits that a certain proportion of these cells did not contain the carbon.

Ferrata might also be considered to have applied biological criteria when he used the behavior of the mononuclear cells to supra-vital stains as a method of classification. But Ferrata (1908) believed that the supra-vital staining furnished strong evidence for the derivation of all the mononuclear cells from lymphatic tissue, and considered that morphological differences were due to ageing of the cells. Ferrata's evidence may be summarized as follows:

(1) The cells form a complete series; all intermediate sizes are present in the blood.

(2) As the cells become larger the nucleus stains less intensely.

(3) Metachromatic droplets (Brilliant Cresyl Blue) appear only in the larger, older cells.

(4) The collection of Brilliant Cresyl Blue in "plasmasomes" is common to all members of this group and distinguishes these cells from the polymorphous cells.

(5) Fat droplets occur in all the mononuclear cells.

(6) The nuclei have similar proportions in all the mononuclear cells.

Ferrata placed his confidence in the existence of but one type of mononuclear cell in the blood on the fact that all intermediate forms would be seen among the living, supra-vitally stained cells. It was therefore of the greatest interest to discover that the supra-vital method furnishes one of the most valuable methods of distinguishing two groups of mononuclear cells in the blood. The method has not only been shown to be a satisfactory one for the study of human blood but has been applied to other mammals, e.g., rabbits, and has been found of far greater value for the identification of transitionals in experimental blood studies in that animal than either Giemsa or oxydase stains.

Ferrata is correct in so far as all intermediate sizes of lymphocytes from small to large can be found. However, there are very few of the lymphocytes which reach a size which is as large as the average size of monocytes.[3] Yet most of the characteristics of these two kinds of mononuclear cells were found to be shared in common, though these characteristics were expressed in various degrees. A few characteristics were observed, however, which were definitely not held in common and which served to separate two types of cells. The cytoplasm of the lymphocytes, both in the smallest and in the largest forms, had a clear hyalin nature, so that the cell appeared distinctly different from the transitional cells with their ground glass or finely granular cytoplasm. This criterion was of minor importance compared with the differences which were found to exist in the segregation apparatus. The segregation apparatus did not furnish an absolute point of separation between polymorphonuclear and mononuclear cells as had been supposed by Ferrata. This set of granules is present in both cell types. Qualitatively the segregation apparatus was alike in all of the mononuclear cells, that is, the rate of accumulation and final size reached by the structures was practically the same in all the mononuclear cells. *The number of granules characteristic of the segregation apparatus of the two cell types is very different.* In the lymphocytes there were one to eight granules, depending on the size of the cell. On the average the segregation apparatus of the lymphocytes consisted of two to three granules. In the transitional cells these granules are always numerous. Forty to sixty granules would be a reasonable estimate of the number always found in this cell type. Since the granules can be seen in fresh unaltered living cells without the use of dyes, and since they are especially clear in dark field observations, these criteria can be applied to separate even unstained living mononuclear cells.[4] These differences are rendered strikingly clear by dyes which accentuate the segregation apparatus such as Neutral Red, Brilliant Cresyl Blue, and Nile Blue Sulfate. These

[3] The term "monocyte" is used in this paper to include both the transitionals and large mononuclears of Ehrlich. This distinction made by Ehrlich was based on nuclear indentation. Hematologists are coming to lay less and less stress on nuclear form in separating the mononuclear cells, and in living cells it is a distinctly unreliable distinction. The living nucleus is continually changing shape. The nuclear form of these large mononuclear cells is particularly motile.

[4] Naegeli clearly recognized the fact that his so-called specific granules of monocytes were not artifacts, for they could be seen in the living cell. It is all the more remarkable that he was not sufficiently impressed with this to urge that the distinction between these cells and the lymphocytes could thus be adequately seen in the living unaltered cell. His method for the recognition of these cells consisted in the azurophilic reaction of the fine monocyte granules in a good Giemsa stain.

facts were not, consequently, overlooked by Hammar in his studies with Brilliant Cresyl Blue, though he looked upon the segregation structures as degenerative products produced by autolysis. It is of distinct interest that the vital stains enable us to deny Naegeli's conception of the peculiar or specific nature of the granules of the transitionals or monocytes, for they show that the granules of monocytes are at any rate essentially the same in nature as those possessed by all blood cells. They consist of mitochondria and of segregation granules and merely the number of the latter is very significantly increased.[5]

The supra-vital application of certain dyes to human blood has been shown to furnish an excellent method for distinguishing the special group of mononuclear cells comprised originally of the transitionals and large mononuclears of Ehrlich—the monocytes. It is of interest that these cells and they alone stand in some sort of relation—as yet not wholly clear—to the endothelial macrophages which may be experimentally produced in the mammalian body by a variety of procedures which will be summarized in a later paper.

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[5] The identity or non-identity of the vitally stained granules with the sporadically occurring, so-called, azurophilic granulation of lymphocytes discovered by Michaelis and Wolff (1902) has interested several observers. Hammar treated a preparation of vitally stained cells with a double May-Grunwald and Giemsa stain (after Pappenheim). He decided that neither in position, size, or number were the two kinds of granules the same. Betances (1918) reached a similar conclusion although Ferrata concluded they were the same structures. Furthermore, most lymphocytes normally show a few segregation bodies, but at best only a portion of them possess the azurophilic granules. Naegeli argues convincingly that the monocytic granules are never brilliant red as are the lymphocytic ones, that they are smaller, and are invariably, not occasionally, present.

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(See footnote, page 1.)

THE EXPERIMENTAL PRODUCTION OF CIRCULATING ENDOTHELIAL MACROPHAGES AND THE RELATION OF THESE CELLS TO THE MONOCYTES

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The great significance of certain large phagocytic cells in the metabolic and protective reactions of the body is just beginning to be known. These cells have been variously called pyrrhol cells (Goldman), adventitia cells (Marchand), rhagiocrine cells (Renaut), resting wandering cells (Maximow), endothelial leucocytes (Mallory), clasmatocytes (Ranvier), histiocytes (Aschoff, Kiyono), macrophages (Evans), Kupfer cells and the other "specific endothelia" in the lymph glands, hemal nodes, bone marrow, and spleen. In the study of these cells endless discussion has arisen on their relationship to other cells and tissues of the body and on the relationships between these cells themselves. Due largely to recent work with vital stains, done first by Bouffard but really inaugurated with greater care by E. E. Goldman and continued by Kiyono and by Evans and Schulemann, we now know that these cells have certain common characteristics, chief of which is the power to receive, store, and concentrate within their protoplasm a large group of coloring matters belonging to the acid azo dyes. Correlative with this behavior is their activity as phagocytes. The idea of classifying these apparently diverse elements in one cell group was first suggested by H. M. Evans (1915), who reëmphasized the early, fundamental immunological work of Metchnikoff in resurrecting the term "macrophage" for the cells of this group. In particular, it would seem unfortunate to adopt the term "clasmatocyte" for the connective tissue members of this cell group, for Ranvier's term, as Maximow has shown, was based on two errors: first, his identification of the mast cells of Amphibia with the connective tissue macrophages of Mammals; and, second, the notion that these cells undergo what he called "clasmatosis," i.e., a pinching off or abstriction of portions of their protoplasm and solution of the same in the tissue juices—a process which does not occur.

The cells would ordinarily be classified as belonging to the connective tissue, to the endothelium or, in rarer instances, to the blood stream. As Goldmann first showed and as has been more fully demonstrated by the work of Evans and Schulemann and of Evans and Scott, they constitute one of the two great groups of connective tissue cells. Furthermore, the cells line the capillaries and the sinusoids in the liver, lymph glands, hemal nodes, bone marrow, and spleen. It is of great interest, moreover, that, in animals subjected to chronic treatment with lithium carmine or benzidine dyes, the capillaries or sinusoids in these five localities do not merely have their endothelial walls densely loaded with the injected substance but also contain large, free, similarly marked cells. These cells have been especially studied by Evans and his collaborators and by Kiyono and Aschoff. Entirely independently of this work and dating back for a considerable number of years, pathologists have recognized that in a variety of diseases large phagocytic cells are developed; and that such cells are not merely interstitially placed but may lie in blood vessels. F. B. Mallory, who early recognized these facts, has termed these cells endothelial leucocytes; Aschoff has designated them histiocytes, and Evans has spoken of them as endothelial macrophages which have become free to join the blood current.

The extent to which these large mononuclear phagocytic cells are found in the actual circulating blood is not as yet known to us; for, being relatively rare and atypical, they have undoubtedly not been recognized or catalogued with any frequency. But a rapidly increasing number of reports of these cells has been appearing in the literature, and we now know that a considerable number of pathological conditions are included in the cases showing this response. Ehrlich very early reported the presence of large phagocytic mononuclears in the case of *paroxysmal haemoglobinuria*. Schilling has reported macrophages in several other conditions. He has made his most careful study of the cells as they appeared in *ulcerative endocarditis* (1919). A case of *Libman's subacute bacterial endocarditis* has been followed in connection with this study and the cell phenomena observed will be discussed later in connection with their bearing on the results of the experimental work.

The presence of these cells in the blood stream of animals which had been injected chronically with various vital dyes, while denied by the early workers (e.g., Goldmann), was noticed by Schulemann, who, however, barely mentioned the fact of their occasional occurrence.

Evans and Winternitz in 1911 again had occasion to notice frequently the presence of these strange, large, brilliantly colored cells in the blood stream in rabbits which had been injected chronically with Trypan Blue for studies on vital staining and milliary tuberculosis. They picked up these cells in the blood from the ear vein. The first careful reports on the presence of these cells in the blood, however, was given by Aschoff and Kiyono (1913) and by Kiyono (1913–14). They found the cells in the circulation after repeated intravenous injections of lithium carmine and established the fact that the cells were relatively rare throughout most of the blood stream but relatively frequent in the veins draining the five organs mentioned above.

From the above account it is clear that work from a number of different sources—above all the histological observations of Mallory and the work with vital carmine or benzidine stains with which Kiyono and Evans may be identified—has all conspired to show clearly that the endothelium in certain particular localities is especially active, creating cellular products which are probably of great importance in the bodily mechanism.

Besides the interesting questions which must now be raised regarding the specific function of these cells, the question of their relation to the other white cells is an important one. It is possible that certain of the normal leucocytes are endothelial derivatives and hence also to be termed "macrophages," although at a lower stage of development than the great cells met with in disease or after the use of vital stains. Patella (1909), for instance, as is well known, though without important confirmation, has urged the derivation of all the mononuclear leucocytes from endothelium. Aschoff and Kiyono jumped to the conclusion that the large, carmine-laden cells which they found in the blood stream in the localities mentioned, in addition to their endothelial origin, were also to be identified with the transitional leucocytes of Ehrlich. They do not discuss the fact that the more normal sized and abundant transitionals throughout the circulation were not so stained, nor do they present any detailed evidence justifying their surmise.

Mallory and McJunkin have not hesitated to identify the group of large mononuclear, or transitional leucocytes of Ehrlich with the unusual, large cells—the true macrophages—and consequently designate transitional leucocytes as endothelial leucocytes. Their criteria for this venture, however, have never been adequate and this fact has naturally led to reserve on the part of other students. At one time

McJunkin fancied that he could, so to speak, mark out these cells in experimental animals by the physiological test of phagocytized intravenously injected carbon; at another time he proposed the oxydase reaction as being equally reliable. These cells are undoubtedly more active than are the other mononuclears, the lymphocytes, in ingesting intravascular particulate matter, but by no means all of them are ever thus concerned. Furthermore, the oxydase reaction is notoriously unreliable, not merely differing in its results in the same material when applied by different hands or in different ways but also, as Menten has shown, differing in different animals. The reaction is often given incompletely at best in the guinea pig and may fail in the case of the rabbit. It must also be stated against McJunkin that the macrophages as a class do not respond to the oxydase reaction.

In spite of the above statements, evidence has recently been accumulating to show that there is nevertheless some sort of relation between the transitionals of Ehrlich and the class of great phagocytic cells. In this connection work in clinical haematology, above all that of Victor Schilling, has shown that in certain conditions transitional leucocytes or monocytes (Naegeli) are specifically increased, giving a true monocytosis; in other conditions the macrophages are abundant without notable increase of the monocytes, giving macrophagocytosis; and in still other conditions the macrophage response is distinctly associated with increase of the monocytes. The latter class of conditions, furnishing an intermediate step or link between the two types of response, would appear to suggest strongly a biological relationship in the two cells.

It is now necessary to comment briefly on the criteria for recognizing the transitionals or monocytes. Until very recently the mononuclear leucocytes could merely be classified as "small" (about the size of a red cell) and "large," and in the latter group would be found the forms approaching considerable dimensions and with concave or horseshoe nuclei. We are now satisfied that the latter cells do not normally possess neutrophilic granulations and that the term "transitional" in Ehrlich's sense is a complete misnomer. The introduction of the Romanovsky methods of staining has, however, shown that the old Ehrlich large mononuclear and transitional group does, in fact, belong together and can be separated from the lymphocytes—this by virtue of the fact that the transitional group to which Naegeli has now applied the term "monocyte" possesses in its cytoplasm a special, fine, dustlike azurophilic granulation. Only those studies on

the blood which have been carried out by means of the best, improved, modern Giemsa stain can lay claim to a justifiable separation of the mononuclear leucocytes into the monocyte and lymphocyte group. The reaction is given beautifully by the blood of man. It is unfortunate that it may be secured only with great difficulty, if at all, in certain mammalia. The monocytes are frequently difficult to recognize in this way with dependability in the rabbit. It is hence of much importance that other methods for detecting this group in the mononuclear blood cells be devised. The writer has previously reported that the method of vital staining by means especially of Neutral Red but also with Brilliant Cresyl Blue, Nile Blue Sulfate and a considerable number of other dyes does furnish such a method. It has hence seemed of much importance for an experimental study to be undertaken in animals in which the monocytes could be recognized with certainty and in which at the same time the production of considerable numbers of true circulating macrophages could be brought about. The present studies undertaken at the suggestion of Professor Evans and with his aid aim to accomplish that task.

Rabbits were submitted to chronic intravenous dosage with various materials and at time intervals of every few days for a total time interval varying from a month to four or five months, the majority of the cases being of the latter duration. In one or two instances animals were treated for a little over a year. Intravenous injections were made of the colloidal dyes Niagara blue 2B and lithium carmine, of the larger colloids and suspensoids constituted by red gold in sodium lysalbinate, india ink and lamp black in gelatin solution, and with certain proteins or split products of proteins, namely, gelatin and sodium lysalbinate. An obvious point in common between the three kinds of stimulating agents is that the solutions injected always contained substances in the colloidal state. Blood was continually studied as obtained in the living animal from the right and left ventricle, and from the ear veins. It is surprising how few macrophages are found in the peripheral blood even after prolonged stimulation of the macrophage producing organs. It is possible to prove that this is due to the interposition of the pulmonary circulation, which filters out, as it were, the great cells, so that the latter do not reach the peripheral blood in appreciable quantities, even when poured into the right heart in enormous numbers. *The method of right ventricular puncture in the living animal, though involving some difficulty, is consequently essential for the discovery of the time and extent of production*

of these cells. In all cases the blood was withdrawn quickly with oiled or paraffined instruments and submitted to a supra-vital stain by a combination of Janus Green and Neutral Red as described previously. Fixed specimens were stained with combined Jenner-Giemsa, Wilson's stain, Graham's oxydase, McJunkin's oxydase and combined benzidine-polychrome. At the time of autopsy similar studies were made both by the method of fixed specimens and by vital stains of living films of blood from all the great veins. As reported by Kiyono, the blood in the splenic and hepatic veins was always richer in macrophages than in other vessels. High counts of these cells in these veins have been paralleled by histological evidence of the direct production of the macrophages by the splenic and hepatic endothelium, as has been shown by Kiyono and Evans.

The difference in the macrophage content of the blood of the two sides of the heart in the living animal may be truly spectacular when the macrophages are abundant, for enormous numbers may exist in the right and almost none in the left ventricle. The method of right ventricular puncture has shown that a hitherto unsuspected massive production of macrophages may occur a short time after administration of the stimulating agent in the course of these long or chronic treatments. Indeed, the right ventricle has been observed when 90 per cent of the leucocytes were macrophages, the left ventricle containing simultaneously less than .1 per cent of the cells. Our method of treatment and examination of the blood was hence ideally adapted to discover the peculiarities of these hitherto rare inhabitants of the blood, and of their possible relationships or transformation from other blood cells, because great numbers of the cells were at our disposal.

One of the chief physiological points coming out of the work has been the demonstration that the macrophages are not present continually even in the venous heart blood of chronically injected animals. *They appear in showers,* i.e., in chronically treated animals the time is finally reached when the animal responds to every intravenous injection of the stimulating agent within a time varying from a few hours to somewhat over a day by pouring forth great numbers of macrophages into the general venous circulation.

There is also an equally interesting abrupt disappearance of macrophages from the circulation and such a phenomenon may occur within a few minutes. The right ventricle may show 70 or 80 per cent of the white cells as great macrophages and a second puncture fifteen minutes

later disclose but 1 or 2 per cent of these cells.[1] In all cases what have been termed typical macrophages were very large cells exceeding the dimensions of any leucocyte, even the largest of the transitionals. Their nuclei were either round, oval, or somewhat indented and a few instances were encountered of cells containing two or even three nuclei. Furthermore, mitotic figures were occasionally observed. The cytoplasm usually contains granules and vacuoles in variable quantities often arranged radially around the centriolar apparatus, and frequently contains phagocytized material in the form of either fragmented red or white cells, as well as some of the stimulating substances which had been injected intravenously. With Giemsa stains the cytoplasm is packed with fine azurophilic granulations. Except for the phagocytized material or foci the cytoplasm was negative in its response to oxydase tests. The cells are beautiful when studied with supra-vital stains. With Brilliant Cresyl Blue the segregation apparatus is violet and variable in amount, the phagocytized material staining very early and intensely. The mitochondria are unstained. The refractive vacuoles and certain non-refractive vacuoles are unstained. With Neutral Red and Janus Green the segregation apparatus and phagocytized material varies from orange to red, while the mitochondria are blue green. It is remarkable that the macrophages do not contain the injected material in appreciable quantities during the greater part of the time in which they are poured into the blood stream. This indicates a great over-production of the cells.

Some other significant changes in the blood seem associated with the production of circulating macrophages. During the time of the showers when the right ventricular content of macrophages was high, the blood from this chamber of the heart would be thick and stringy, contrasting with the thin arterial blood of the left ventricle. The clotting time of this viscuous blod may also be greatly prolonged or a

[1] A striking clinical illustration of the same "shower phenomenon" was fortunately obtained in the medical service of the University of California Hospital during the months of May and June, 1921. These were the two terminal months of the course of a fatal case of Libman's subacute bacterial endocarditis occurring in a young man eighteen years of age. I am indebted to Doctors Moffitt, Kerr, and Sampson for permission to observe and for much aid in the study of this interesting case.

Positive blood cultures were obtained of non-haemolytic streptococcus viridans. Macrophages were found varying from 1 to 1½ per cent in the ear vein blood at various times and on one occasion (June 11) 14 per cent of these great cells were encountered, but on the same day, four hours later, less than 1 per cent of the cells were present. There were occasions when none of the cells could be detected in the blood and in all respects the spasmodic or showerlike behavior of the macrophages seen in experimental animals was encountered here in man.

clot indeed fail to form when similar simultaneous left ventricular samples clot normally. Furthermore, the peculiar right ventricular blood has a reduction or practical disappearance of platelets. In all cases differential white counts were made by examining at least two hundred cells in the living supra-vitally stained films. In this way it was possible to gain an accurate idea of the behavior of that interesting group of normal mononuclear blood cells whose relationship to the macrophages was sought, namely, the monocytes.

The supra-vital method shows a striking parallelism in the behavior of macrophages and monocytes, a parallelism not shown by the lymphocytic cells. *But of greater importance is the fact that during the time of occurrence of the so-called macrophage showers, all intermediate cell types are encountered, bridging the gap between normal monocytes and macrophages.* It is furthermore of interest that, with many animals, shortly preceding macrophage showers the monocytes were observed to increase numerically, and during the shower were to some extent filtered out by the lungs just as the macrophages are almost completely filtered out by the pulmonary circulation. This evidence would appear at any rate to now point convincingly to a kinship between the two kinds of cells.

Inasmuch as the monocytes, however, are not effectively filtered and circulate generally, a very rapid production of the monocytes in any region would be necessary to appreciably raise the monocyte count of blood from those regions and hence throw light in this way on the sites of origin of these cells. Such significant differences were not observed.

These studies appear to give the first certain indication of the relationship between the monocyte and macrophage, and yet if the same mother endothelium is supposed to proliferate these two types of cell, we must regard macrophage production as a pathological or atypical response. Certain it is that the macrophages can not usually be regarded as the result merely of further growth of the monocyte cell, for there would then probably appear far greater numbers of these only slightly hypertrophied monocytes in the general circulation. This is never the case. During the prolonged treatments of our animals and between the showers of macrophages, the monocytes always constitute a distinct cell group with not conspicuously larger members even though their numbers may be increased. It does not appear now so remarkable that macrophages are not more frequently encountered in many conditions since we know of the filtration by the lungs.

Furthermore, the splenic blood from several normal animals has been examined and seems to always contain at least a few true macrophage cells. It is important for us to realize that in addition to these facts which indicate that macrophages are not merely transformed monocytes, there are other facts which indicate certain differences, as well as the affinities on which we have already dwelt, between these two groups of cells. Mention here must be made of the fact that the monocytes, contrary to the opinion of Aschoff and Kiyono, in animals treated chronically with any of these agents, do not contain the agent in question. They are not stained vitally by the benzidine or carmine dye, and, while macrophages are sometimes not so stained, the reintroduction of the vital dye into the blood stream of the living animal will result in the staining of the macrophages but not of the monocytes.

While the present work, then, may be said to point strongly to the biological affinities of these two interesting cell types, it cannot be said to show an identity or that one may be transformed into the other in the free or circulating blood stream. This evidence is in consonance with all that we know about a similar lack of transformation of one type of leucocyte to another in the blood current, our information tending to bring the conviction that a cell once shed into the stream thenceforth usually undergoes no significant transformation.

Transmitted September 6, 1921.

(See footnote, page 1.)

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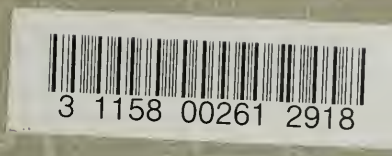
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